USE OF A PEPTIDE THAT INTERACTS WITH ALPHA V BETA3 INTEGRIN OF ENDOTHELIAL CELL

TECHNICAL FIELD

The present invention relates to a peptide having an angiogenesis-inhibitory effect, and more particularly, to the anti-angiogenic use of a peptide that interacts with the αvβ3 integrin of endothelial cells.

BACKGROUND ART

Angiogenesis is defined as the formation of new capillary blood vessels from preexisting micro-vessels. Normal angiogenesis occurs during embryogenic development, tissue remodeling, organ growth, wound healing and female reproductive cycles (corpus luteum development) under tight physiological regulation (Folkman and Cotran, Int. Rev. Exp. Patho., 16:207-248, 1976). Generally, angiogenesis involves the proteolysis of the blood vessel basement membrane by proteases, followed by the migration, proliferation and differentiation of endothelial cells to form tubules and eventually the regeneration of new blood vessels.

Unregulated and abnormal angiogenesis may lead to various diseases. Examples of angiogenesis-related diseases that occur in pathological conditions include various cancers(tumors); vascular diseases such as vascular malformation, arteriosclerosis, vascular adhesions, and edematous sclerosis; ocular diseases such as corneal graft neovascularization, neovascular glaucoma, diabetic retinopathy, angiogenic corneal disease, macular degeneration, pterygium, retinal degeneration, retrolental fibroplasia and granular conjunctivitis; inflammatory diseases such as

rheumatoid arthritis, systemic Lupus erythematosus and thyroiditis; and dermatological diseases such as psoriasis, capillarectasia, pyogenic granuloma, seborrheic dermatitis and acne (USA Patent No. 5,994,292; Korean Patent Application Laid-Open No. 2001-66967; D'Amato R. J. *et al.*, Ophtahlmol., 102:1261-1262, 1995; Arbiser J. L. J. Am. Acad. Derm., 34(3):486-497, 1996; O'Brien K. D. *et al.*, Circulation, 93(4):672-682, 1996; Hanahan D. *et al.*, Cell, 86:353-364, 1996).

Thus, studies on the mechanism of angiogenesis and the discovery of substances capable of inhibiting angiogenesis are of significant importance in the prevention and treatment of various diseases, including cancer. Current studies on the inhibition of angiogenesis are being performed on target genes by various strategies, including a strategy of administering a competitive substance to inhibit the action of VEGF and bFGF (basic fibroblast growth factor), which are known as potent inducers of angiogenesis, and a strategy of regulating the expression of integrin in vascular endothelial cells to inhibit the metastasis of the cancer cells. Regarding the relationship of angiogenesis with cancer, studies on the correlation between vascular absorption and angiogenesis induced by cancer cells and on proteins that induce angiogenesis are being performed but are still large incomplete. Studies on angiogenic inhibition are applicable to the diagnosis, treatment and/or prevention of a variety of angiogenesis-related diseases, and thus, there is a continued need for research and development regarding angiogenesis.

Meanwhile, βig-h3 that is an extracellular matrix protein was first isolated by differential screening of a cDNA library made from a human lung adenocarcinoma cell line (A549) that had been treated with TGF-β1 (Skonier J. *et al.*,

DNA Cell Biol., 11:511-522, 1992). The βig-h3 protein consists of 683 amino acids and contains an amino-terminal secretory sequence and a carboxy-terminal RGD (Arg-Gly-Asp) motif serving as a ligand recognition site for several integrins (Skonier, J. *et al.*, DNA Cell Biol., 11:511, 1992). Also, the βig-h3 protein contains four homologous internal repeat domains (designated "fas-1 domains") which are homologous to similar motifs in the *Drosophila* fasciclin-I protein. Such fas-1 domains have highly conserved sequences found in the secretory and membrane proteins of many organisms, including mammals, insects, sea urchins, plants, yeast, and bacteria (Kawamoto T., *et al.*, Biochim. Biophys. Acta., 288-292, 1998). Each of the fas-1 domains consists of 110-140 amino acids and comprises two highly conserved branches of about 10 amino acids (H1 and H2).

The βig-h3 protein is known to have a fibrillar structure and to interact with several extracellular matrix proteins such as fibronectin and collagen (Kim J.-E., *et al.*, Invest. Ophthalmol. Vis. Sci., 43:656-661, 2002). Furthermore, the βig-h3 protein has been reported to be involved in cell growth and differentiation, and wound healing and morphogenesis (Skonier J., *et al.*, DNA Cell Biol., 13:571-584, 1994; Dieudonne S. C., *et al.*, J. Cell. Biochem., 76:231-243, 1999; Kim J.-E., *et al.*, J. Cell. Biochem., 77:169-178, 2000; Rawe I. M., *et al.*, Invest. Ophthalmol. Vis. Sci., 38:893-900, 1997; and LeBaron R. G., *et al.*, J. Invest. Dermatol., 104:844-849, 1995). In addition, the βig-h3 protein is known to mediate the adhesion of many different cell types, including corneal epithelial cells, chondrocytes and fibroblasts (LeBaron R. G., *et al.*, J. Invest. Dermatol., 104:844-849, 1995; Ohno S., *et al.*, Biochim. Biophys. Acta, 1451: 196-205, 1999; and Kim J.-E., *et al.*, J. Biol. Chem., 275:30907-30915, 2000). However, there is still no report indicating that the βig-h3 protein is involved in angiogenesis.

DISCLOSURE OF THE INVENTION

Accordingly, the present inventors have performed extensive studies to determine whether the βig-h3 protein is involved in angiogenesis, and consequently, found that YH motif conserved in the fas-1 domains of the βig-h3 protein shows an anti-angiogenic effect through the αvβ3 integrin of endothelial cells, thereby completing the present invention.

Therefore, an object of the present invention is to provide the novel use of a peptide that interacts with the αvβ3 integrin of endothelial cells.

To achieve the above object, in one aspect, the present invention provides a method of inhibiting endothelial cell adhesion, endothelial cell migration and/or angiogenesis, comprising administering to a subject in need thereof an effective amount of a peptide that interacts with the αvβ3 integrin of endothelial cells.

In another aspect, the present invention provides a method of treating or preventing angiogenesis-related diseases, comprising administering to a subject in need thereof an effective amount of a peptide that interacts with the αvβ3 integrin of endothelial cells.

In yet another aspect, the present invention provides a pharmaceutical composition for the inhibition of angiogenesis or for the treatment or prevention of angiogenesis-related diseases, comprising as active ingredient a peptide that interacts with the αvβ3 integrin of endothelial cells.

In still another aspect, the present invention provides the use of a peptide interacting with the αvβ3 integrin of endothelial cells, for the preparation of a

pharmaceutical agent for the inhibition of endothelial cell adhesion, endothelial cell migration and/or angiogenesis.

In another further aspect, the present invention provides the use of a peptide interacting with the αvβ3 integrin of endothelial cells, for the preparation of an agent for the treatment or prevention of angiogenesis-related diseases.

As used herein, the term "YH motif" is defined as an amino acid sequence comprising tyrosine-histidine (Y-H) or asparagine-histidine (N-H) residues highly conserved in the fas-1 domains of a βig-h3 protein, and flanking several hydrophobic amino acid residues adjacent to the conserved residues(e.g., leucine and isoleucine) (Kim, J.-E. *et al.*, J. Biol. Chem., 277:46159-46465, 2002). The YH motif is also highly conserved in fas-1 domains derived from other proteins in addition to the βig-h3 protein (see Fig. 1).

As used herein, the term "effective amount" is defined as an amount at which the effect of inhibiting endothelial cell migration, endothelial cell adhesion and/or angiogenesis is shown.

As used herein, the term "subject" means animals, including mammals, particularly human beings. The subject may preferably be a patient who requires treatment.

Herein after, the present invention will be described in detail.

A peptide according to the present invention may consist of at least 18 amino acids, comprising tyrosine-histidine (Y-H) or asparagine-histidine (N-H) residues, and at least three hydrophobic amino acids with bulky side chains. The hydrophobic amino acids with bulky side chains may be leucines (L) or isoleucines

(I). The inventive peptide comprises at least three of the hydrophobic amino acids, preferably four to six of them. The hydrophobic amino acids are preferably adjacent to the tyrosine-histidine (Y-H) or asparagines-histidine (N-H) residue. Concretely, the hydrophobic amino acids may be located at one side (N-terminal or C-terminal region) or both sides of the tyrosine-histidine (Y-H) or asparagines-histidine (N-H) residues.

Concretely, the inventive peptide may have an amino acid sequence comprising the YH motif which is conserved in the fas-1 domains. The inventive peptide may preferably consist of at least 18 amino acids, comprising the YH motif derived from each of the fas-1 domains of the βig-3 protein. More preferably, the inventive peptide may comprises an amino acid sequence represented by (I, D, E or K)-(E, A or Q)-L-(L, R or A)-(N, D or S)-(A, L, K or I)-(L or Y)-(R, N, L or K)-(Y or N)-H-(M, I or G)-(V, L, Q or G)-(G, K, T or D)-(R, S, L or E)-(R, A, E or I)-(V, M, T or L)-(L, C or V)-(T, A, G or S). The amino acid abbreviations as described above have the following definitions: I, isoleucine; D, aspartate; E, glutamate; K, lysine; A, alanine; Q, glutamine; L, leucine; R, arginine; N, asparagine; S, serine; Y, tyrosine; H, histidine; M, methionine; G, glycine; V, valine; T, threonine; and C, cysteine. Most preferably, the inventive peptide may comprise an amino acid sequence selected from the group consisting of SEQ ID NO: 23 to SEQ ID NO: 26. The amino acid sequence of SEQ ID NO: 23 is derived from the first fas-1 domain of the βig-h3 protein, and the amino acid sequence of SEQ ID NO: 24 from the second fas-1 domain of the βig-h3 protein, the amino acid sequence of SEQ ID NO: 25 from the third fas-1 domain of the βig-h3 protein, and the amino acid sequence of SEQ ID NO: 26 from the fourth fas-1 domain of the βig-h3 protein.

It is understood that functional equivalents or salts of the peptide consisting of at least 18 amino acids, comprising tyrosine-histidine (Y-H) or asparagine-histidine (N-H) residues and at least three hydrophobic amino acids with bulky side chains, are within the scope of the inventive peptide. As used herein, the term "functional equivalents" is defined as peptides where some amino acids in the inventive peptide are mutated to such a degree that they do not influence the physiological activity of the peptides. In other words, the peptides having the same or similar structure as the inventive peptide as well as amino acid sequences are within the scope of the present invention insofar as they show substantially the same physiological activity as that of the inventive peptide. As used herein, the term "physiological activity" means the activity of inhibiting endothelial cell adhesion, endothelial cell migration and/or angiogenesis by the interacting with the $\alpha v\beta 3$ integrin of endothelial cells.

The mutation as described above includes the substitution, deletion and/or addition of amino acids, and may be preferably the substitution of amino acids. An example of this mutation is the case where the hydrophobic amino acids with bulky side chains adjacent to the tyrosine-histidine (Y-H) or asparagine-histidine (N-H) on the YH motif are substituted with other amino acids, and preferably serine. More preferably, the inventive peptide may also have either an amino acid sequence selected from the group consisting of SEQ ID NO: 12 to SEQ ID NO: 14, or an amino acid sequence selected from the group consisting of SEQ ID NO: 18 to SEQ ID NO: 20, which comprises the amino acid sequence selected from SEQ ID NO: 12 to SEQ ID NO: 14.

The mutation may also be the case where the tyrosine-histidine (Y-H) or asparagine–histidine (N-H) on the YH motif is substituted with two other amino

acids. Preferably, the tyrosine (Y) or asparagine (N) may be substituted with one selected from the group consisting of serine (S), histidine (H), phenylalanine (F) and threonine (T), and/or the histidine may be substituted with asparagine (N). More preferably, the tyrosine-histidine (Y-H) or asparagine–histidine (N-H) may be substituted with amino acids selected from the group consisting of serine-histidine (S-H), histidine-histidine (H-H), phenylalanine-histidine (F-H), threonine-histidine (T-H) and tyrosine-asparagine (Y-N), which are conserved in the fas-1 domains of proteins known in the prior art (see Fig. 1).

Moreover, the tyrosine-histidine (Y-H) or asparagine–histidine (N-H) may also be substituted with hydrophobic amino acids, and preferably alanine-alanine (A-A). In this case, the inventive peptide may have either an amino acid sequence of SEQ ID NO: 11, or an amino acid sequence of SEQ ID NO: 17, which comprises the amino acid sequence of SEQ ID NO: 11.

In addition, the mutation also includes the case where both the tyrosine-histidine (Y-H) or asparagine–histidine (N-H) and the flanking hydrophobic amino acids with bulky side chains are substituted as described above. In this case, however, the hydrophobic amino acids with bulky side chains are preferably present at least three within the inventive peptide. Preferably, the inventive peptide may have either an amino acid sequence of SEQ ID NO: 15 or SEQ ID NO: 16, or an amino acid sequence of SEQ ID NO: 21 or SEQ ID NO: 22, which comprises the amino acid sequence of SEQ ID NO: 15 or SEQ ID NO: 16.

Furthermore, the scope of the functional equivalents according to the present invention also encompasses peptide derivatives obtained by partially modifying the chemical structure of the inventive peptide while maintaining the backbone and physiological activity of the inventive peptide. Examples thereof

include structural modifications to modify the stability, storage, volatility and solubility of the inventive peptide.

The inventive peptide may be easily prepared by a chemical synthesis method known in the art (Creighton, Proteins; Structures and Molecular Principles, W. H. Freeman and Co., NY, 1983). Typical methods includes but are not limited to liquid or solid state synthesis, fragment condensation, and F-MOC or T-BOC chemistry (Chemical Approaches to the Synthesis of Peptides and Proteins, Williams *et al.*, Eds., CRC Press, Boca Raton Florida, 1997; A Practical Approach, Athert on & Sheppard, Eds., IRL Press, Oxford, England, 1989).

Moreover, the inventive peptide may be prepared by a genetic engineering method. For this purpose, a DNA sequence encoding the inventive peptide is constructed according to a conventional method. The DNA sequence can be constructed by PCR-amplification with suitable primers. Alternately, the DNA sequence may also be synthesized by any standard method known in the art, for example, using an automated DNA synthesis system (sold from Biosearch or Applied Biosystems). The constructed DNA sequence is inserted into a vector containing one or more expression control sequences (e.g., promoter and enhancer, etc.) which are operatively linked to the DNA sequence to control the expression of the DNA sequence. Host cells are then transfected with the resulting recombinant expression vector. The transfected cells are incubated in a suitable medium and condition to express the DNA sequence, and a substantially pure peptide encoded by the DNA sequence is recovered. The peptide recovery may be performed by a method known in the art (e.g., chromatography). As used herein, the term "substantially pure peptide" means that the inventive peptide does not substantially contain any peptides derived from host cells. For the genetic engineering method

for synthesizing the inventive peptide, reference may be made to the following publications: Maniatis *et al.*, Molecular Cloning; A laboratory Manual, Cold Spring Harbor laboratory, 1982; Sambrook *et al.*, supra; Gene Expression Technology, Method in Enzymology, Genetics and Molecular Biology, Method in Enzymology, Guthrie & Fink (eds.), Academic Press, San Diego, Calif, 1991; and Hitzeman *et al.*, J. Biol. Chem., 255:12073-12080, 1990.

Previously, the present inventors reported that the βig-h3 protein has both α3β1 integrin-interacting motif that induces the adhesion of epithelial cells (Kim, J.-E. *et al.*, J. Biol. Chem., 275:30907-30915, 2000), and αvβ5 integrin-interacting motif that mediates the adhesion of fibroblasts (Kim, J.-E. *et al.*, J. Biol. Chem., 277:46159-46165, 2002). Then, in the present invention, whether the βig-h3 protein and its fas-1 domains have endothelial cell adhesion activity was examined, and an integrin receptor that is involved in the adhesion of endothelial cells by the βig-h3 protein was identified. Furthermore, to discover a motif within the βig-h3 protein that interacts with the integrin receptor, deletion mutants of the βig-h3 protein were prepared and their fragments, which are involved in cell adhesion, were examined (see Examples 1 to 4-1).

The results showed that the βig-h3 protein and each of its fas-1 domains mediate the adhesion of endothelial cells at almost equal activity with each other (see Fig. 2), and that the αvβ3 integrin is involved in the adhesion of endothelial cells by interacting with the βig-h3 protein (see Figs. 3a to 3d). Furthermore, it was found that amino acids 548-614 (ΔH1H2(6)) of SEQ ID NO: 1, which corresponds to a fragment of the fas-1 domains, have activity related to the cell adhesion (see Fig. 4b).

Meanwhile, the present inventors previously reported that an YH motif that binds to the αvβ5 integrin was present within a fragment corresponding to amino acids 548-614 of the βig-h3 protein (Kim, L. E. *et al.*, J. Biol. Chem., 277:46159-46165, 2002). Thus, to confirm that the YH motif mediates endothelial cell adhesion by interacting with the αvβ3 integrin, the present inventors constructed a fragment of 29 amino acids (amino acids 560-588 of SEQ ID NO: 1) comprising the YH motif, and relevant mutants whose tyrosine-histidine residues and flanking several isoleucines/leucines were substituted with alanine-alanine residues and serines, respectively, and examined their cell adhesion activity (see Example 4-2).

The results revealed that although various mutants of the YH motif have somewhat different activities, they all retain the activity of mediating the cell adhesion (see Fig. 4b).

Then, on the basis of the sequence of each fas-1 domain of the βig-h3 protein, the present inventors synthesized four peptides (YH18 synthetic peptides represented by SEQ ID NOs: 23 to 26) consisting of 18 amino acids, which were designed to comprise the YH motif. The effect of the synthesized peptides on the adhesion of endothelial cells was tested (see Example 5).

The results showed that the YH18 synthetic peptides inhibit endothelial cell adhesion, which is mediated by the βig-h3 protein, in a dose-dependent manner (see Fig. 5). This suggests that YH motif that is a conserved sequence in the fas-1 domains is responsible for endothelial cell adhesion to βig-h3 protein through the αvβ3 integrin.

Furthermore, in the present invention, whether or not the YH motif is involved in the migration of endothelial cells in addition to the adhesion of endothelial cells was tested (see Fig. 7). The results revealed that the YH18 synthetic peptide inhibits endothelial cell migration, which is promoted by the βig-h3 protein, in a dose-dependent manner (see Figs. 7a and 7b).

Additionally, in the present invention, whether or not the YH motif that inhibits endothelial cell adhesion and migration shows an anti-angiogenic effect was tested (see Example 8). The results showed that angiogenesis is effectively inhibited by the YH18 synthetic peptide both in vivo and in vitro (see Figs. 8a and 8b).

The peptide according to the present invention has the following physiological activities:

First, the inventive peptide interacts with the αvβ3 integrin of endothelial cells.

Second, it inhibits the adhesion and migration of endothelial cells.

Third, it inhibits angiogenesis both *in vitro* and *in vivo*.

Accordingly, the present invention provides a pharmaceutical composition for the inhibition of endothelial cell adhesion, endothelial cell migration and/or angiogenesis, comprising the inventive peptide as an active ingredient. Also, the present invention provides a pharmaceutical composition for the treatment or prevention of angiogenesis-related diseases, comprising the inventive peptide as an active ingredient.

The angiogenesis-related diseases that can be treated or prevented according to the present invention include various cancers(tumors); vascular diseases such as hemangioma, angiofibroma, vascular malformation, arteriosclerosis, vascular adhesions, and edematous sclerosis; ocular diseases such as corneal graft neovascularization, neovascular glaucoma, diabetic retinopathy, angiogenic corneal disease, macular degeneration, pterygium, retinal degeneration, retrolental fibroplasia and granular conjunctivitis; inflammatory diseases such as rheumatoid arthritis, systemic Lupus erythematosus and thyroiditis; and dermatological diseases, such as psoriasis, capillarectasia, pyogenic granuloma, seborrheic dermatitis and acne (USA Patent No. 5,994,292; Korean Patent Application Laid-Open No. 2001-66967; D'Amato R. J. *et al.*, Ophtahlmol., 102:1261-1262, 1995; Arbiser J. L. J. Am. Acad. Derm., 34(3):486-497, 1996; O'Brien K. D. *et al.*, Circulation, 93(4):672-682, 1996; Hanahan D. *et al.*, Cell, 86:353-364, 1996). More preferred examples include cancers, arthritis, psoriasis, diabetic eye diseases, arteriosclerosis, and inflammation.

The pharmaceutical composition comprising the inventive peptide as an active ingredient may further comprise a pharmaceutically acceptable carrier, for example, a carrier for oral or parenteral administration. Examples of the carrier for oral administration include lactose, starch, cellulose derivatives, magnesium stearate, and stearic acid. For oral administration, the inventive peptide may be mixed with an excipient and used in various forms, including enteric tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups and wafers. Also, examples of the carrier for parenteral administration includes water, suitable oils, saline solution, aqueous glucose and glycol, and the inventive composition may further comprise stabilizers and conservatives. Suitable examples of the stabilizers include

antioxidants, such as sodium hydrogensulfite, sodium bisulfite and ascorbic acid. Suitable examples of the preservatives include benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol. For other pharmaceutically acceptable carriers, reference may be made to the following literature: Remington's Pharmaceutical Sciences, 19th ed., Mack Publishing Company, Easton, PA, 1995.

The pharmaceutical composition according to the present invention may be formulated in various forms for oral or parenteral administration. The formulations for parenteral administration are typically injection formulations, and preferably isotonic aqueous solution or suspension. The injection formulations may be prepared using suitable dispersing or wetting agents, and suspending agents, according to any technique known in the art. For example, the components may be formulated for injection by dissolving them in a saline or buffer solution. Examples of the formulations for oral administration include tablets and capsules, and these formulations may comprise diluents (e.g., lactose, dextrose, sucrose, mannitol, sorbitol, cellulose, and/or glycin) and lubricants (e.g., silica, talc, stearic acid and magnesium or calcium salts thereof, and/or polyethylene glycol), in addition to the active ingredient. The tablets may comprise binders such as magnesium aluminum silicate, starch paste, gelatin, tragacanth, methyl cellulose, sodium carboxymethylcellulose and/or polyvinylpyrrolidone, and occasionally, it may further comprise disintegrants such as starch, agar, alginate or a sodium salt thereof, absorbing agents, coloring agents, flavoring agents and/or sweetening agents on a case by case basis. These formulations may be prepared by a conventional method such as mixing, granulation or coating.

The pharmaceutical composition according to the present invention may additionally comprise aids such as preservatives, wettable powders, emulsifiers, salts

for the regulation of osmotic pressure, and/or buffers, and other therapeutically useful materials. The pharmaceutical composition may be formulated according to a conventional method.

The total amount of the inventive peptide as an active ingredient in the inventive pharmaceutical composition can be administered to a subject as a single dose over a relatively short period of time, or can be administered using a fractionated treatment protocol where multiple doses are administered over a prolonged period of time. Although the content of the inventive peptide in the inventive pharmaceutical composition can vary depending on the severity of diseases, the inventive peptide can be generally administered several times a day at a dose of 10 μg-10 mg. However, the dose of the inventive peptide depends on many factors, including the age, weight, general health, sex, disease severity, diet and excretion of a subject, as well as the route of administration and the number of treatments to be administered. In view of these factors, any person skilled in the art would adjust the particular dose so as to obtain an effective dose for inhibiting angiogenesis, or for treating or preventing angiogenesis-related diseases. The pharmaceutical composition according to the present invention is not specifically limited in its formulation, administration route and administration mode insofar as it shows the effects of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the comparison between amino acid sequences which contain the tyrosine-histidine residues that are highly conserved in fas-1 domains derived

from various proteins, and the conserved leucine/isoleucine residues adjacent to the tyrosin-histidine residues.

Fig. 2 is a schematic diagram (A) showing recombinant proteins containing each fas-1 domain of a βig-h3 protein, and a graphic diagram (B) showing the adhesion of HUVECs to the plate coated with βig-h3 or each of its fas-1 domains, in terms of absorbance.

BSA: bovine serum albumin as control

Wild-type: recombinant βig-h3 His-β-b protein containing all of four fas-1 domains.

D-I: first fas-1 domain

D-II: second fas-1 domain

D-III: third fas-1 domain

D-IV: fourth fas-1 domain

Fig. 3a is a graphic diagram showing the inhibition of HUVECs adhesion to the βig-h3 coated on the plate by function-blocking antibodies against various integrins.

BSA: plate coated with BSA

None: no treatment

α3: treated with P1B5 (antibody to α3)

α5: treated with P1D6 (antibody to α5)

αv: treated with P3G8 (antibody to αv)

β1: treated with 6S6 (antibody to β1)

β3: treated with B3A (antibody to β3)

αvβ3: treated with LM609 (antibody to αvβ3)

αvβ5: treated with P1F6 (antibody to αvβ5)

Fig. 3b is a graphic diagram showing the inhibition of HUVECs adhesion to each of fas-1 domains coated on the plate by a function-blocking antibody against αvβ3 or αvβ5 integrin.

BSA: plate coated with BSA

D-I: first fas-1 domain of βig-h3

D-II: second fas-1 domain of βig-h3

D-III: third fas-1 domain of βig-h3

D-IV: fourth fas-1 domain of βig-h3

Fig. 3c shows the results of FACS analysis for the expression of integrin on the HUVECs surface using a function-blocking antibody against αvβ3 or αvβ5 integrin.

Fig. 3d shows the results of dose-dependent Western-immunoblotting analysis for the binding ability of biotin-βig-h3 to a HUVECs cell membrane (A), and for the inhibition of biotin βig-h3 binding to the HUVECs cell membrane by a function-blocking antibody against αvβ3 or αvβ5 integrin (B), in which the concentration-dependent Western-immunoblotting analysis is performed to identify a receptor for βig-h3 that is involved in endothelial cell adhesion. β-tubulin is an internal control for equal protein loading.

Fig. 4a is a schematic diagram showing various deletion mutants of the fourth fas-1 domain (D-IV) of βig-h3.

Fig. 4b schematically shows various substitution mutants of an YH motif conserved in the fourth fas-1 domain, and graphically shows test results for the HUVECs adhesion activity of the mutants.

Black blocks: substituted amino acids.

Fig. 5 shows the amino acid sequences of YH18 synthetic peptides derived from each fas-1 domain of βig-h3, and graphically shows the dose-dependent inhibition of HUVECs adhesion to βig-h3 by the peptides.

Fig. 6a is a graphic diagram showing the adhesion of HEK293 cells transfected with a β3 integrin expression vector to βig-h3.

pc/293: HEK293 cells transfected with pcDNA3 vector

β3/293: HEK293 cells transfected with β3 integrin expression vector

Fig. 6b is a graphic diagram showing the inhibition of β3/293 cell adhesion to βig-h3 coated on the plate by a function-blocking antibody against αvβ3 or αvβ5 integrin.

BSA: plate coated with BSA

IgG: treated with mouse IgG

αvβ3: treated with LM609 (antibody to αvβ3)

αvβ5: treated with P1F6 (antibody to αvβ5)

Fig. 6c is a graphic diagram showing the dose-dependent inhibition of β3/293 cell adhesion to βig-h3 coated on the plate by YH18 synthetic peptides.

Fig. 7a is a photograph (A) and a graphic diagram (B), which show the inhibition of HUVECs migration toward βig-h3 coated on the plate by a function-blocking antibody against αvβ3 or αvβ5 integrin.

BSA: plate coated with BSA

IgG: treated with mouse IgG

αvβ3: treated with LM609 (antibody to αvβ3)

αvβ5: treated with P1F6 (antibody to αvβ5)

Fig. 7b is a photograph (A) and a graphic diagram (B), which show the inhibition of HUVECs migration toward βig-h3 coated on the plate by an YH18 synthetic peptide.

BSA: plated with BSA

control: treated with 5% DMSO

YH18-con.: treated with YH18-con. peptide (control peptide)

YH18-500μM: treated with 500 μM YH18 synthetic peptide

YH18-1mM: treated with 1 mM YH18 synthetic peptide

Fig. 8a is a photograph (A) showing the inhibition of HUVECs tube formation by an YH18 synthetic peptide, and a graphic diagram (B) showing the result of measurement for the number of tube branches formed.

Control: treated with 5% DMSO

YH18-con.: treated with YH18-con. peptide (control peptide)

YH18-500μM: treated with 500 μM YH18 synthetic peptide

YH18-1 mM: treated with 1 mM YH18 synthetic peptide

Fig. 8b is a photograph (A) showing the inhibition of angiogenesis by the YH18 synthetic peptide, a photograph (B) showing a section stained with H&E, and a graphic diagram (C) showing the result of measurement for the number of blood vessels.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention will be described in detail by examples. It will however be obvious to a person skilled in the art that the present invention is not limited to or by the examples.

Example 1: Expression and purification of βig-h3 protein and its fas-1 domains

1-1: Construction of expression vector

Previously, the present inventors reported recombinant proteins comprising βig-h3 and each of its fas-1 domains (Kim, J.-E. *et al.*, J. Biol. Chem., 275:30907-30915, 2000; and Korean Patent Registration No. 10-0382042). Thus, βig-h3 and its fas-1 domains were prepared in the same manner as described in the report.

Concretely, a recombinant human βig-h3 protein (hereinafter, referred to as "βig-h3 His-β-b") that expresses all of four fas-1 domains was prepared using a pHis-β-b vector. The pHis-β-b vector had been prepared by inserting an *Asp*718-*Bgl*II fragment (obtained by partially deleting the amino terminal region of βig-h3 cDNA) into the *Eco*RV/*Eco*RI sites of pET-29β. Also, to express recombinant proteins containing each fas-1 domain of human βig-h3, the present inventors PCR-amplified four cDNA fragments of βig-h3, which include the first fas-1 domain (βig-h3 D-I), the second fas-1 domain (βig-h3 D-II), the third fas-1 domain (βig-h3 D-III) or the fourth fas-1 domain (βig-h3 D-IV), respectively (see A of Fig. 2).

Then, each of the PCR products was cloned into the *Eco*RV/*Xho*I sites of a pET-29b(+) vector (Novagen; Madison, WI). The constructed expression vectors were named "pβig-h3 D-I", "pβig-h3 D-II", "pβig-h3 D-III" and "pβig-h3 D-IV", respectively. The amino acid sequences of the four fas-1 domains of βig-h3 are represented by SEQ ID NO: 2 to SEQ ID NO: 5, respectively.

1-2: Transformation of E. coli and purification of recombinant protein

E. coli BL21 (DE3) cells were transformed with each of the expression vectors constructed in Example 1-1. The transformed *E. coli* cells were incubated in LB medium containing 50 μg/ml kanamycin. To induce the expression of each recombinant protein, when the absorbance of the culture reached 0.5-0.6 at a 595 nm, the culture was added with 1 mM IPTG (isopropyl-β-D-(-)-thiogalactopyranoside) and further incubated at 37 °C for three hours. Next, purification of the expressed proteins was performed according to the method described by Kim, J.-E. *et al.*, J. Cell. Biochem., 77:169-187, 2000. For this purpose, the cells were collected by centrifugation and resuspended in buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 0.5 mM DTT). The cell suspension was disrupted by sonication. The proteins expressed in the form of an inclusion body were dissolved in an 8M urea-denaturation buffer, and the denaturated proteins were purified with a Ni-NTA resin (Qiagen). The recombinant proteins were eluted in 200 mM imidazole solution, and purified by dialysis against 20 mM Tris-HCl buffer containing 50 mM sodium chloride in a stepwise manner from high to low urea concentration. The expressed and purified proteins were analyzed by SDS-PAGE (data not shown). Unlike the recombinant protein βig-h3 His-β-b containing all of the four fas-1 domains, the recombinant proteins containing each of the four fas-1 domains were synthesized in a water-soluble form and thus did not require a modification step. Also, they could be easily obtained in large amounts.

Meanwhile, the E. coli transformed with the expression vectors pHis-β-b, pβig-h3 D-II and pβig-h3 D-IV were termed "*E. coli* BL21/His-β-b", "*E. coli* BL21/Hisβ-g" and "*E. coli* BL21/Hisβ-e", respectively, and deposited in the Korean Collection for Type Cultures (KCTC), Korean Research Institute of Bioscience and

Biotechnology, under accession numbers KCTC 18008P, KCTC 18010P and KCTC 18009P, respectively, on April 25, 2000.

Example 2: Test of endothelial cell adhesion activity of βig-h3 and its fas-1 domains

Cell adhesion assay was performed according to the method described by Kim, J.-E. *et al.*, J. Biol. Chem., 277:46159-46165, 2002. For this purpose, 10 μg/ml of each of the recombinant proteins (βig-h3 His-β-b, βig-h3 D-I, βig-h3 D-II, βig-h3 D-III and βig-h3 D-IV) prepared in Example 1 was placed into a flat-bottomed 96-well ELISA (enzyme-linked immunosorbent assay) plate (Costar, Corning Inc., NY) and then incubated overnight at 4 °C, to coat the surface of the plate. As a control, 2% BSA was coated on the plate. Then, the plate was treated with PBS (phosphate-buffered saline) containing 2% BSA, and blocked at room temperature for one hour.

Meanwhile, HUVECs (human umbilical vein endothelial cells; Clonetics, San Diego, CA) were incubated in EGM medium (Clonetics, San Diego, CA) containing 2% FBS (Fetal Bovine Serum) under a condition of 37 °C and 5% CO_2. The incubated cells were suspended in medium at a density of 3×10^5 cells/ml, and 0.1 ml of the cell suspension was added to each well of the plate. Next, the cells were incubated at 37 °C for 30 minutes and washed one time with PBS buffer to remove cells which had not been attached to the plate. Attached cells were added with 50 mM citrate buffer (pH 5.0) containing 3.75 mM p-nitrophenyl-N-acetyl β-D-glycosaminide and 0.25% Triton X-100, and incubated at 37 °C for one hour. Thereafter, 50 mM glycine buffer (pH 10.4) containing 5 mM EDTA was added to block the activity of the enzyme. The absorbance was measured at a 405-nm in a

Bio-Rad model 550 microplate reader. Here, the higher the number of cells adhered to the plate, the higher the absorbance.

The results showed that, as shown in B of Fig. 2, βig-h3 mediated the adhesion of endothelial cells, and each of the fas-1 domains of βig-h3 also mediated the adhesion of endothelial cells with an almost equal activity to that of βig-h3.

Example 3: Identification of integrins that are involved in adhesion of endothelial cells to βig-h3

3-1: Test 1 for identification of integrin receptors

To identify integrins that are involved in the adhesion of endothelial cells to βig-h3, the present inventors have performed a cell adhesion inhibition assay using various antibodies that specifically blocks the function of integrins.

For this purpose, 5 μg/ml of monoclonal antibodies specific to different types of integrin (Chemicon, International Inc, Temecula, CA) was preincubated at 37 °C for 30 minutes with HUVECs in 0.1 ml of the cell suspension (3×10^5 cells/ml). The following antibodies were used in this test: P1B5 (antibody to α3), P1D6 (antibody to α5), P3G8 (antibody to αv), 6S6 (antibody to β1), B3A (antibody to β3), LM609 (antibody to αvβ3) and P1F6 (antibody to αvβ5). A culture which had not been preincubated with the antibody was used as a control. Then, the incubated cells were transferred onto plates precoated with the recombinant protein βig-h3 His-β-b and incubated at 37 °C for 30 minutes. The attached cells were then quantified in the same manner as in Example 2. The results showed that, as shown in Fig. 3a, the adhesion of endothelial cells to βig-h3 was inhibited specifically by the antibodies to αvβ3 integrin and β3 integrin, but it was not inhibited by the antibodies to other integrins, including α3 and α5.

3-2: Test 2 for identification of integrin receptors

In Example 2 above, it was confirmed that βig-h3 and also its fas-1 domains mediate the adhesion of endothelial cells. Thus, in order to identify an integrin receptor for each of the fas-1 domains of βig-h3, the present inventors coated a plate surface with each of the fas-1 domains of βig-h3 and performed a cell adhesion inhibition assay.

The results showed that, as shown in Fig. 3b, the adhesion of endothelial cells to each of the fas-1 domains was inhibited specifically by the antibody to αvβ3, but it is not inhibited by the antibody to αvβ5.

3-3: Confirmation of integrins that are expressed on surface of endothelial cells

To confirm that HUVECs express both αvβ3 and αvβ5 integrin on their surface, the present inventors performed an FACS analysis using monoclonal antibodies specific to the two integrins.

For this purpose, a plate in which HUVECs had been grown to confluence was treated with PBS buffer containing 0.25% trypsin and 0.05% EDTA to detach the cells from the plate surface. The cells were washed two times with PBS buffer and resuspended in PBS buffer. The cell suspension was added with an anti-αvβ3 integrin antibody (LM609; Chemicon, International Inc, Temecula, CA) or an anti-αvβ5 integrin antibody (PIF6; Chemicon, International Inc, Temecula, CA) and incubated at 4 °C for one hour. The cells were then further incubated for one hour at 4 °C with 10 μg/ml of a FITC-conjugated secondary goat antimouse IgG antibody (Santa Cruz Biotechnology, Inc., CA). The resulting cells were analyzed at 488 nm

on the flow cytometer FACSCalibur system (Becton Dickinson, San Jose, CA) equipped with a 5-watt laser. A control was incubated with a secondary antibody alone.

The results showed that, as shown in Fig. 3c, HUVECs expressed both the αvβ3 integrin and the αvβ5 integrin. However, the expression level of the αvβ5 integrin was far less than that of the αvβ3 integrin.

3-4: Test 3 for identification of integrin receptors

To confirm that HUVECs adhesion that is mediated by βig-h3 depends on αvβ3 integrin, the present inventors tested the binding affinity of βig-h3 in the presence of an antibody that specifically blocks the function of the integrin. This binding assay was performed according to the method described by Maile, L. A., *et al.*, J. Biol. Chem., 275:23745-23750, 2002.

First, HUVECs were suspended in medium at a density of 1 x 10^5 cells/ml. 1 ml of the cell suspension was preincubated with anti-αvβ3 antibody (LM609) or anti-αvβ5 antibody (P1F6) at 37 °C for 30 minutes. Thereafter, the preincubated cells were incubated with biotinylated βig-h3 (hereinafter, referred to as "biotin-βig-h3") in a serum-free medium containing 0.1% BSA at 4 °C for 5 hours. The biotin-βig-h3 was added at concentrations of 1 x 10^{-10}, 1 x 10^{-9} and 5 x 10^{-9} μM, respectively. Then, the cells were washed three times with PBS buffer (pH 7.4), and dissolved at 4 °C in ice-cold buffer A (10mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.5% SDS, 0.02% sodium azide, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were clarified by centrifugation at 13,000 rpm for 30 minutes at 4 °C. Equal amounts of protein were

then separated by SDS-PAGE, 8% gel. The amount of biotin-βig-h3 was determined by Western immuno-blotting.

To visualize the biotin-βig-h3, the membranes were incubated with HRP (hoseradish peroxidase; Amersham Biosciences)-conjugated streptavidin. Next, Binding of the peroxidase-labeled antibody was visualized using ELC (enhanced chemiluminesence; Amersham Biosciences). As an internal control, a β-tubulin protein was subjected to Western blotting to verify equal protein loading.

The results showed that βig-h3 was bound to HUVECs surface in a dose-dependent manner (see A of Fig. 3d), and its binding was specifically inhibited only by the antibody to αvβ3 integrin (see B of Fig. 3d).

Such results suggest that each of the fas-1 domains of βig-h3 contains a motif that mediates the adhesion of endothelial cells through the 'αvβ3 integrin', but not the αvβ5 integrin.

Example 4: Identification of βig-h3 motif interacting with αvβ3 on endothelial cell adhesion

4-1: Identification of αvβ3 integrin-interacting motifs using deletion mutants of fas-1 domains

To identify motifs that interact with the αvβ3 integrin when βig-h3 or its fas-1 domains induce the adhesion of endothelial cells by interacting with the αvβ3 intergrin, the present inventors prepared deletion mutants of the fas-1 domains and tested their cell adhesion activity.

For this purpose, deletion mutants of the fas-1 domains were prepared according to the method described by Kim, J.-E. *et al.*, J. Biol. Chem., 275:30907-

30915, 2000. Concretely, in the present invention, there were prepared several deletion mutant fragments which lack a H1 or H2 peptide, which is highly conserved in the fas-1 domains, and/or an EPDIM motif, which is involved in the adhesion of corneal epithelial cells by interacting with the α3β1 integrin (see Fig. 4a). Information of the deletion mutant fragments is given in Table 1 below. The amino acid region of each of the deletion mutant fragments is based on the amino acid sequence of βig-h3, which is represented by SEQ ID NO: 1.

Table 1: Deletion mutants of fas-1 domains

No.	Amino acid region of deletion mutant fragment	Designation	Characteristic	SEQ ID NO
1	548-632	ΔH1	5'-terminal fragment containing H1 peptide was deleted.	6
2	502-620	ΔH2	H2 peptide was deleted.	7
3	502-614	ΔH2(6)	EPDIM and H2 peptide were deleted.	8
4	548-620	ΔH1H2	5'-terminal fragment containing H1 peptide, and H2 peptide, were deleted.	9
5	548-614	ΔH1H2(6)	5'-terminal fragment containing H1 peptide, H2 peptide and EPDIM, were deleted.	10

Each of the deletion mutants was generated by PCR using a cDNA template encoding the fourth fas-1 domain (SEQ ID NO: 5) (Kim, J.-E. *et al.*, J. Biol. Chem., 275:30907-30915, 2000). Each of the PCR-amplified DNA fragments was cloned into the *Eco*RV/*Xho*I sites of the pET-29b(+) vector (Novagen; Madison, WI). The mutations were confirmed by sequence analysis, and the deletion mutants were expressed and purified according to a prior method (Kim, J.-E., *et al.*, J. Cell. Biochem., 77:169-178, 2000). Thereafter, the cell adhesion activity of each of the deletion mutants was tested in the same manner as in Example 2.

The results showed that ΔH1H2(6), which is the smallest deletion mutant fragment, still retains endothelial cell adhesion activity (data not shown). This suggests that αvβ3 integrin-interacting motif is present within a fragment corresponding to amino acids 548-614 of SEQ ID NO: 1.

4-2: Identification of αvβ3 integrin-interacting motif using YH motif mutants

Previously, the present inventors reported that an YH motif, which contains tyrosine-histidine residues highly conserved in the fragment corresponding to amino acids 548-614 of SEQ ID NO: 1, and several leucine and isoleucine residues adjacent to the tyrosine-histidine residues, binds to the αvβ5 integrin (Kim, J.-E., *et al.*, J. Biol. Chem., 277:46159-46165, 2002). Thus, the present inventors suspected that the YH motif may also interact with the αvβ3 integrin to mediate endothelial cell adhesion. For this reason, in the present invention, the tyrosine-histidine residues conserved in the fas-1 domains, and/or the leucine and isolucine residues adjacent to the tyrosine-histidine residues, were substituted in various combinations by alanine or serine (Kim, J.-E., *et al.*, J. Biol. Chem. 277:46159-46165, 2002)(see Fig.

4b). The amino acid sequences of the prepared YH motif mutants are shown in SEQ ID NO: 17 to SEQ ID NO: 22. The mutations were confirmed by sequence analysis. Then, the substitution mutants were expressed and purified according to a known method (Kim, J.-E., *et al.*, J. Cell. Biochem., 77:169-178, 2000). The substitution mutants were tested for their cell adhesion activity according to the same manner as in Example 2.

The results shown in Fig. 4b confirmed that, in a case of D-IV-AA where the tyrosine-histidine residues highly conserved in the fas-1 domains were substituted with alanine-alanine residues that are hydrophobic amino acids, and in cases of D-IV-L and D-IV-R where the leucine and isoleucine residues adjacent to either side (N- or C-terminal region) of the tyrosine-histidine residues were substituted with a hydrophilic amino acid, serine, cell adhesion activity was equal to those of the fas-1 domain (D-IV) and its fragment (H1H2(6)). Meanwhile, in a case of D-IV-LYHR where all of the isoleucine and leucine residues present in both side of the tyrosin-histidine residues were substituted with serine, cell adhesion activity was reduced to half that of the fas-1 domain. Also, in cases of D-IV-LAA and D-IV-AAR where both the tyrosine-histidine residues and the isoleucine and leucine residues adjacent to either side of the tyrosine-histidine residues were substituted with the alanine-alanine residues and the serin residue, respectively, cell adhesion activity was somewhat lower than those of D-IV-LAA and D-IV-AAR. However, all the substitution mutants showed a higher cell adhesion activity than that of a control. Theses results suggest that not only the tyrosine-histidine residues but also the hydrophobic amino acids with bulky side chains, which are adjacent to both sides of the tyrosine-histidine residues, are required for the adhesion of endothelial cells.

Example 5: Assay of inhibition of endothelial cell adhesion by YH18 synthetic peptide

To further confirm that the YH motif within βig-h3 is involved in endothelial cell adhesion, the present inventors performed cell adhesion assay using YH18 synthetic peptides (Kim, J.-E., *et al.*, J. Biol. Chem., 277:46159-46165, 2002). The YH18 synthetic peptides, which are derived from the YH motif conserved in all the fas-1 domains of βig-h3, are peptides of 18 amino acids containing tyrosine (or asparagine)-histidine residues and flanking several leucine and isoleucine residues.

Concretely, in this Example, the following YH18 synthetic peptides were used: a YH18 synthetic peptide of SEQ ID NO: 23 derived from the first fas-1 domain of βig-h3 (hereinafter, referred to as "D-I YH18"), a YH18 synthetic peptide of SEQ ID NO: 24 derived from the second fas-1 domain (hereinafter, referred to as "D-II YH18"), a YH18 synthetic peptide of SEQ ID NO: 25 derived from the third fas-1 domain (hereinafter, referred to as "D-III YH18"), and a YH18 synthetic peptide of SEQ ID NO: 26 derived from the fourth fas-1 domain (hereinafter, referred to as "D-IV YH18") (see Fig. 5). As a control peptide, an YH18-con. peptide of SEQ ID NO: 27 was used. All the above peptides had been synthesized by AnyGen Co. Ltd, Kwangju, Korea. Thereafter, the present inventors tested whether the YH18 synthetic peptides have the ability to inhibit endothelial cell adhesion in the wells coated with βig-h3.

For this purpose, HUVECs were suspended in medium at a density of 3×10^5 cells/ml, and each of the YH18 synthetic peptides and the YH-con. peptide was added to 0.1 ml of the cell suspension at concentrations of 100 μM, 300 μM, 500 μM and 1,000 μM, respectively. The peptide-containing cell suspensions were

preincubated at 37 °C for 30 minutes. The preincubated cells were added to each well of a 96-well plate precoated with a recombinant βig-h3 His-β-b protein. Then, the cells were tested in the same manner as in Example 2.

The results showed that, as shown in Fig. 5, the adhesion of endothelial cells, which is mediated by βig-h3, is inhibited by the YH18 synthetic peptides derived from the fas-1 domains of βig-h3, in a dose-dependent manner. This suggests that the YH motifs are involved in endothelial cell adhesion to βig-h3 through the αvβ3 integrin.

Example 6: Reconfirmation of functional receptor αvβ3 of endothelial cells for βig-h3

To reconfirm that the αvβ3 integrin mediates the adhesion of endothelial cells to βig-h3, a cell adhesion assay was performed using HEK293 cells which had been stably transfected with a human β3 integrin expression vector.

6-1: Cell adhesion assay

To construct a human β3 integrin expression vector, RT-PCR was performed using a human placenta poly(A)$^+$ RNA as a template, thereby generating a 2.4-kb β3 cDNA (Chandrika, S. K. *et al.*, J. Biol. Chem., 272:16390-16397, 1997). The amplified β3 cDNA was digested with *Hin*dIII/*Xba*I, and then cloned into the pcDNA3 vector (Invitrogen). The resulting vector was named "β3/pcDNA3". Then, 1 μg of the β3/pcDNA3 vector was introduced into HEK293 cells (ATCC, catalog No. CRL 1573) using lipofectamin (Gibco). A control was introduced with a pcDNA3 vector containing no human β3 cDNA. Since all the vectors contain a G418 selection marker, the stable transfected cells were screened using 1 mg/ml of G418. The cell transfected with the β3/pcDNA3 vector was

named "β3/293", and the cell transfected with the pcDNA3 vector (control) was named "pc/293". Each of the screened transfectants was incubated in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS, streptomycin and penicillin. Then, cell adhesion assay was performed in the same manner as in Example 2.

The results showed that, as shown in Fig. 6a, the β3/293 cells were strongly adhered to βig-h3, whereas the pc/293 cells were not adhered to βig-h3, in a similar manner to the plate coated with BSA. This is because the pc/293 cells do not synthesize β3 while producing only αv by themselves and thus do not adhered to βig-h3, but the β3/293 cells can express β3 to produce the αvβ3 integrin.

6-2: Assay of inhibition of endothelial cell adhesion using integrin function-blocking antibodies

In the present invention, to further confirm that βig-h3 mediates endothelial cell adhesion through the αvβ3 integrin, cell adhesion assay using antibodies that block the function of integrins was performed in the same manner as in Example 3-1.

The results showed that, as shown in Fig. 6b, the adhesion of β3/293 cells to βig-h3 was inhibited specifically by a function-blocking antibody against the αvβ3 integrin, whereas the adhesion is not inhibited by an antibody to αvβ5.

6-3: Assay of inhibition of endothelial cell adhesion using YH18 synthetic peptide

In Example 5, the present inventors confirmed that the YH18 synthetic peptides inhibit endothelial cell adhesion to βig-h3 through the αvβ3 integrin. Then, the present inventors tested whether the YH18 synthetic peptide also inhibits the

adhesion of β3/293 cells to βig-h3. The test was performed in the same manner as in Example 5.

The results showed that, as shown in Fig. 6c, the YH18 synthetic peptides derived from each of the fas-1 domains inhibit the adhesion of β3/293 to βig-h3 in a dose-dependent manner.

From the above results, it was reconfirmed that the YH motif in each fas-1 domain of βig-h3 mediates endothelial cell adhesion through αvβ3, and a peptide containing the YH motif inhibits the endothelial cell adhesion by interacting with the αvβ3 integrin.

Example 7: Assay of inhibition of endothelial cell migration by YH18 synthetic peptide

7-1: Assay of inhibition of endothelial cell migration using integrin function-blocking antibody

Firstly, to examine whether βig-h3 is involved in the migration of endothelial cells, the present inventors performed cell migration assay. The cell migration assay was performed in a transwell plate (8 μm pore size, Costar, Cambridge, MA). The undersurface of the membrane was coated with the recombinant βig-h3 His-β-b protein (10 μg/ml) prepared in Example 1 at 4 °C, and then, blocked for 1 hour at room temperature with PBS buffer containing 2% BSA. Meanwhile, HUVECs were added with an anti-αvβ3 antibody (LM609) or an anti-αvβ5 antibody (P1F6) and preincubated at 37 °C for 30 minutes. A control was added with mouse IgG. Then, the HUVECs preincubated with the antibody were suspended in medium at a density of 3×10^5 cells/ml, and 0.1 ml of the cell

suspension was added to the upper compartment of the filter. The cells were allowed to migrate at 37 °C for 6-8 hours.

Migration was terminated by removing the cells from the upper compartment of the filter with a cotton swab. The filters were fixed with 8% glutaraldehyde and stained with crystal violet. The extent of cell migration was determined by light microscope, and within each well, cell counting was done in nine randomly selected fields HPF (Microscopic high power fields, x200).

The results showed that the migration of HUVECs was enhanced in those transwells whose undersurface was coated with βig-h3 (data not shown) and this effect was inhibited specifically by an antibody to the αvβ3 integrin but not an antibody to the αvβ5 integrin (see Fig. 7a).

7-2: Assay of inhibition of endothelial cell migration by YH18 synthetic peptide

Then, the present inventors examined whether the migration of endothelial cells is inhibited by the YH18 synthetic peptide. For this purpose, a test was performed in the same manner as in Example 7-1 except that 500 μM or 1 mM of an YH18 synthetic peptide represented by SEQ ID NO: 26 instead of the integrin function-blocking antibody was added together in adding the HUVECs suspension to the upper compartment of the membrane. A control to the peptide treatment was treated with 5% DMSO (solvent), and a control to the YH synthetic peptide added with an YH18-con. peptide represented by SEQ ID NO: 27.

The test results showed that, as shown in Fig. 7b, the YH18 synthetic peptide also inhibited the migration of endothelial cells toward βig-h3. This result suggests that the YH motif of βig-h3 mediates endothelial cell migration through the αvβ3

integrin, and the peptide containing the YH motif inhibits endothelial cell migration by interacting with the αvβ3 integrin.

Example 8: Assay of angiogenesis inhibition by YH18 synthetic peptide

8-1: Endothelial tube formation assay

To examine whether the YH motif in the fas-1 domain inhibits angiogensis, the present inventors assayed the effect of the peptide on endothelial tube formation.

First, 100 μl of Matrigel (Chemicon, International Inc, Temecula, CA) was added to each well of a 96-well plate and allowed to polymerize. HUVECs were suspended in medium at a density of 3×10^5 cells/ml, and 0.1 ml of the cell suspension was added to each well of the well plate coated with Matrigel. At this time, 500 μM or 1 mM of the YH18 synthetic peptide represented by SEQ ID NO: 26 was added together. A control to peptide treatment was treated with 5% DMSO (solvent), and a control to the YH18 synthetic peptide was treated with the YH18-con. peptide represented by SEQ ID NO: 27. Thereafter, the cells were incubated at 37 °C for 16-18 hours. The cells were photographed, and endothelial tubes were counted and averaged.

The results showed that, as shown in Fig. 8a, the YH18 synthetic peptide selectively inhibited the dose-dependent manner. The IC_{50} of the YH18 synthetic peptide was 500 μM.

8-2: Matrigel Plug assay

In the present invention, the angiogenesis-inhibitory effect of the YH18 synthetic peptide, which had been proven *in vitro* assay according to Example 8-1, was assayed *in vivo*. An *in vivo* Matrigel plug assay was performed according to

the method described by Maeshima, Y. *et al.*, J. Biol. Chem., 275:23745-23750, 2000. 5-6 week old male C57/BL6 mice purchased from Hyochang scientific company, Korea, were used.

First, Matrigel (BD Biosciences, MA) was mixed with 20 units/ml heparin, 150 ng/ml bFGF (basic fibroblast growth factor, R&D system, International, Inc), and a YH18 synthetic peptide represented by SEQ ID NO: 26. As a control to the YH18 synthetic peptide, an YH18-con. peptide was used, and a control to peptide treatment was treated with 5% DMSO (solvent). The Matrigel mixture was injected subcutaneously to the C57/BL6 mice. After 7 days, the mice were sacrificed, and the Matrigel plugs were removed and fixed in 4% paraformaldehyde. The Matrigel plugs were buried in paraffin, sectioned and stained with H&E. Their sections were examined by light microscope, the number of blood vessels from 4-6 HPF (high power fields; x200) was counted and averaged. Each group consisted of 3 or 4 Matrigel plugs.

The results showed that a significant reduction in the number of blood vessels was observed at 500 μM of the YH18 synthetic peptide and a complete inhibition of angiogenesis was observed at 1 mM of the peptide (see A of Fig. 8b). Also, the result of observation of the section was the same as described above (see B and C of Fig. 8b).

These results suggest that the peptide containing the YH motif inhibits angiogenesis both *in vitro* and *in vivo*.

Application Example 1: Cancers

Angiogenesis is an essential stage in the growth and metastasis of cancer

cells (Weidner, N. *et al.*, *N. Engl. J. Med.*, 324:1-8, 1991). Tumors are supplied with nutrients and oxygen necessary for their growth and proliferation through new blood vessels, and also new blood vessels invaded by tumors provides an opportunity for cancer cells to enter the blood circulation, thereby causing the metastasis of the cancer cells (Folkman and Tyler, *Cancer Invasion and Metastasis*, Biologic mechanisms and Therapy (S. B. Day ed.), Raven press, New York, 94-103, 1977; Polverini P. J. *Critical Reviews in Oral Biology*, 6(3):230-247, 1995). If angiogenesis does not occur, the tumors will remain in a resting state and will no longer grow (Folkman and Tyler, *Cancer Invasion and Metastasis*, Biologic mechanisms and Therapy (S. B. Day ed.), Raven press, New York, 94-103, 1977). However, as angiogenesis in cancer tissues develops, cancer cell metastasis toward other tissues occurs (Weidner, N. *et al.*, *N. Engl. J. Med.*, 324:1-8, 1991). The metastasis of cancer cells by blood flow rarely occurs through preexisting blood vessels but mainly occurs at sites where angiogenesis actively occurs. In other words, cancer cells flow out through the incomplete walls of blood vessels, or flows out through the basement membrane of blood vessel walls when the basement membrane is degraded by the action of protease, thereby causing systemic metastasis. In some cases of systemic metastasis, endothelial cells being proliferated cause cancer cells to directly migrate into new blood vessels, thereby causing systemic metastasis. Accordingly, the inventive composition for the inhibition of angiogenesis, which contains the YH motif-containing peptide as an active ingredient, has an excellent angiogenesis inhibitory effect, and thus, is highly effective in the prevention of metastasis and the treatment of various cancers.

Application Example 2: Arthritis

Arthritis is an autoimmune disorder. However, a chronic inflammation, which is formed in the synovial cavity between joints during the progression of arthritis, induces angiogenesis to destroy cartilages. Arthritis includes infectious arthritis, degenerative arthritis, rheumatoid arthritis, and arthritis caused by femoral head avascular necrosis, ankylosing spondylitis and congenital malformation. Regardless of the cause of arthritis, the chronic inflammation formed in the synovial cavity between joints during the progression of arthritis is known to induce angiogenesis. It is characterized in that new capillary vessels invade joint to cause damage to cartilages (Kocb A. E. *et al., Arth. Rheum.*, 29:471-479, 1986; Stupack D. G. *et al., J. Med. Biol. Rcs.*, 32:578-281, 1999; Koch A. E., *Arthritis Rheum.*, 41:951-962, 1998). In this case, it has been reported that an inflammatory response, which occurs in several steps depending the kind of diseases to destroy cartilages, plays an important role in the progression of the disease, and the formation of angiogenesis into joints acts as an important pathological mechanism (Colville-Nash, P.R. *et al., Ann. Rheum. Dis.*, 51, 919-925, 1992; Eisenstein, R., *Pharmacol. Ther.*, 49:1-19, 1991). For the treatment of arthritis, it is preferred to inhibit pains and inflammations so as to reduce the destruction rate of joints or muscles and minimize loss of their function. Accordingly, the inventive composition for the inhibition of angiogenesis is highly effective in the prevention of arthritis progression and in the treatment of arthritis.

Application Example 3: Psoriasis

Psoriasis is a skin disease that involves papules and silver white scars. It is generally a chronic proliferative disorder whose deterioration and improvement are repeated. Also, its cause is not yet identified, but it is known that the formation of

new blood cells on pathological lesions or non-lesions, and also the infiltration of immune cells, such as neutrophil, as a result of an increase in blood vessel permeability, play an important role in the deterioration of psoriasis (Bhushan, M. *et al., Br. J. Dermatol.*, 141:1054-1060, 1999). In normal persons, keratinocytes are proliferated one time a month, but in patients with psoriasis, keratinocytes are proliferated one time a week. Since much blood is necessary for this frequent proliferation, angiogenesis will necessarily occur fast (Folkman J. *J. Invest. Dermatol.*, 59:40-48, 1972). Accordingly, the inventive composition for the inhibition of angiogenesis is effective in the treatment of psoriasis.

Application Example 4: Diabetic eye diseases

Ophthalmic diseases by which several million persons each year in the world lose their sight are also caused by angiogenesis (Jeffrey M. I. *et al., J. Clin. Invest.*, 103:1231-1236, 1999; Stupack D. G. *et al., J. Med. Biol. Rcs.*, 32:578-281, 1999). Typical examples of the ophthalmic diseases include age-related macular degeneration(AMD), diabetic retinopathy, retinopathy of prematurity, neovascular glaucoma, and corneal diseases caused by angiogenesis (Adamis A. P. *et al., Angiogenesis,* 3:9-14, 1999). Among them, the diabetic eye disease is one of main diabetic complications capable of causing loss of eyesight, and occurs in a patient with long diabetic duration regardless of the regulation of blood glucose. With a recent improvement in diabetic therapy, the lifespan of diabetic patients is extended while diabetic retinopathy shows a tendency to increase. Thus, the diabetic retinopathy is the leading cause of adult blindness in Western Europe and also Korea. The diabetic retinopathy develops due to the functional reduction of retinal circulation so that angiogenesis spreads along the internal surface and posterior

hyaloid membrane of the retina while blood vessels invade the hyaloid, or bleeding occurs, resulting in blindness. Particularly, it has been reported that diabetic eye diseases, such as diabetic retinopathy, are caused by rapid progression of angiogenesis (Favard, C. *et al., Diabetes, Metab.*, 22:268-273, 1996). Accordingly, the inventive composition for the inhibition of angiogenesis is highly effective in the prevention and treatment of diabetic eye diseases.

Application Example 5: Arterial sclerosis

Sclerosis of the arteries means diseases where arterial walls become thicker to lose their elasticity. It is classified into three morphological categories, the most frequent and important category of which is atherosclerosis caused by the formation of atheroma in the arteries. The atheroma, which is formed of cholesterol and cholesterol ester and enclosed in a fibrous membrane, covers the tunica intima of the arteries while the lumen of arterial walls becomes narrower to block the blood flow of distal organs, thereby causing ischemic injury to the organs. If the atheroma is formed in the main artery, it then weakens the arterial walls to cause aneurysm, blood vessel disruption and thrombosis. In this case, it has been reported that the formation of new blood vessel within atheroma (angiogenesis) plays an important role in weakening the blood vessel walls (Hoshiga, M. *et al., Circ. Res.*, 77:1129-1135, 1995; Kahlon, R. *et al., Can. J. Cardiol.*, 8:60-64, 1992; George, S.J., *Curr. Opin. Lipidol.*, 9:413-423, 1998). Accordingly, the inventive composition for the inhibition of angiogenesis is highly effective in the prevention of severe complications that can be caused by arterial sclerosis.

Application Example 6: Inflammation

Inflammation, which is a response of a living tissue to injury, can be caused by various factors, such as infection and trauma, but show substantially similar changes regardless of its cause and response tissue. Such changes include an increase in blood flow, an increase in permeability of blood vessel walls, and the infiltration of white blood cells, in which all the changes are known to be caused by angiogenesis (Jackson, J. R. *et al., FASEB, J.*, 11:457-465, 1997). Although inflammation is a repairing mechanism of injury and thus not a harmful response, it can cause the injury and deformation of tissues when it excessively occurs or inappropriately occurs as in autoimmune diseases. In regulating such an excessive or inappropriate inflammatory response, the inventive composition for the inhibition of angiogenesis is effective.

INDUSTRIAL APPLICABILITY

As described above, the peptide according to the present invention interacts with the αvβ3 integrin of endothelial cells, so that it inhibits the adhesion and migration of endothelial cells and also shows an excellent angiogenesis-inhibitory effect. Accordingly, the inventive peptide is useful for the inhibition of endothelial cell adhesion, endothelial cell migration, and/or angiogenesis. In addition, it is useful for the treatment or prevention of various angiogensis-related diseases.